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Increased SK3 expression in DM1 lens cells leads to impaired growth through a greater calcium-induced fragility

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Although cataract is a characteristic feature of myotonic dystrophy type 1 (DM1), little is known of the underlying mechanisms. We generated four lens epithelial cell lines derived from DM1 cataracts and two from age-matched, non-DM cataracts. Small-pool PCR revealed typical large triplet repeat expansions in the DM1 cells. Furthermore, real-time PCR analysis showed reduced *SIX5* expression and increased expression of the Ca^{2+} -activated K^+ channel *SK3* in the DM1 cells. These cells also exhibited longer population doubling times which did not arise through reduced proliferation, but rather increased cell death as shown by increased release of lactate dehydrogenase (LDH). Using $^{86}\text{Rb}^+$ as a tracer for K^+ , we found no difference in the resting K^+ influx or efflux kinetics. In all cases, the ouabain sensitive component of the influx contributed ~50% of the total. However, stimulating internal Ca^{2+} by exposure to ionomycin not only caused greater stimulation of K^+ (^{86}Rb) efflux in the DM1 cells but also induced a higher rate of cell death (LDH assay). Since both the hyper-stimulation of K^+ efflux and cell death were reduced by the highly specific SK inhibitor apamin, we suggest that increased expression of SK3 has a critical role in the increased Ca^{2+} -induced fragility in DM1 cells. The present data, therefore, both help explain the lower epithelial cell density previously observed in DM1 cataracts and provide general insights into mechanisms underlying the fragility of other DM1-affected tissues.

INTRODUCTION

The classic adult onset form of myotonic dystrophy (DM) typically presents in midlife as a slowly progressive muscular dystrophy with accompanying myotonia. However, symptoms are not limited to skeletal muscle and in fact a very high proportion of DM patients develop pre-senile cataracts in early adulthood (1). Type 1 (DM1) is the most common form and is associated with the expansion of a CTG-CAG repeat in the 3' untranslated region of the DM protein kinase gene (*DMPK*) and in the promoter of the downstream homeodomain transcription factor gene, *SIX5*. The repeat is polymorphic in the general population, varying from 5 to ~30 repeats, and expanded beyond 50 repeats in patients. There is an inverse correlation with the age of onset and repeat length, with mildly affected patients

typically inheriting 50–100 repeats, adult onset patients 200–400 repeats and congenitally affected children >700 and frequently as many as 1000 repeats.

In addition to being unstable in the germline, the DM1 CTG-CAG repeat is also unstable in somatic tissues, and high levels of somatic mosaicism are observed. Somatic mosaicism accumulates throughout the lifetime of the individual through multiple small gains in a process that is expansion-biased, allele length-dependent and highly tissue-specific (2). Notably, the repeat length in the skeletal muscle cells of adult onset DM1 patients is much larger than is present in their white blood cells and is typically well in excess of 2000 repeats (3).

Why the development of cataracts is such a characteristic feature of DM is unclear. Interestingly, in contrast to skeletal

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muscle symptoms, and despite inheriting relatively large expansions, congenitally affected children do not present with cataracts at birth and do not usually develop them until after 10 years of age (1). The early stages of DM cataract have a characteristic form, with dust-like and iridescent opacities appearing in the outermost cortical fibres. Abe *et al.* (4) also found that there was a greatly reduced density of epithelial cells in DM1 cataracts.

There are currently three main hypotheses to explain how the CTG repeat expansion might cause the diverse symptoms of DM1: first, a reduction in expression of *DMPK*; secondly, a reduction in expression of *SIX5* and thirdly a gain of function by the mutant *DMPK* mRNA (5). Although, there is support for all three, it is reduced expression of *SIX5* in the lens that is most often proposed as the mechanism to explain the prevalence of cataract in DM1. The most compelling evidence for this is that heterozygous *SIX5* knock-out mice develop cataracts (6,7). For either reduced *DMPK* expression or toxic gain of function of *DMPK* mRNA to play a role, *DMPK* must be expressed in the lens. The evidence for this, however, is contradictory (8,9), and whether or not *SIX5* haplo-insufficiency is the primary cause of cataract in DM1 remains an open question.

It has also been shown that increased expression of the Ca^{2+} -activated K^+ channel SK3 (10) has a role in causing the symptoms of myotonia, as injection of the highly specific SK inhibitor apamin (11) into affected muscle reduced the electrical activity associated with myotonia in DM patients (12). SK3 is normally not expressed in adult skeletal muscle but is expressed at high levels in DM-affected muscle cells (13,14). We have recently found evidence that SK channels play a role in modulating electrical events following Ca^{2+} signalling activation in the normal lens (15).

Here we report data obtained from cell lines generated from DM1 cataracts that relate changes in gene expression of *SIX5* and *SK3* to fundamental physiological processes underlying cell survival. We show that increased expression of SK channels leads to increased cell fragility in conditions of Ca^{2+} overload.

RESULTS

Unstable expanded CTG repeats in DM1 patient-specific lens cell lines

In order to determine the repeat length present in the lens cell lines and monitor its stability during tissue culture, we performed Small Pool-PCR analysis of repeat length variation at the DM1 locus in DNA derived from each cell line. Analysis of repeat length variation in the control cell lines, CCAT1 and CCAT2, confirmed that neither cell line contained an expansion at the DM1 locus and that, as expected, the small normal alleles they possessed were stable (data not shown). In contrast, analysis of repeat length variation in the four DM patient-derived lens cell lines revealed the presence of large unstable expanded alleles at all three time points sampled (Fig. 1A and Table 1). As has been previously observed in establishing cell lines from tissues with high levels of repeat length variation (16), all of the cultures at early passage number contained multimodal distributions of

repeat length. These modes most likely represent the clonal expansion of a small number of virally immortalized founder cells in each population. Surprisingly, even the smallest modes comprised cells containing alleles of >400 repeats, and all cultures contained a mode of >1000 repeats in the earliest passage sampled. These data strongly suggest that the lens cells from which these cell lines were derived contained large expanded alleles, with a high proportion of cells with alleles >1000 repeats *in vivo*. At later passages, the repeat length in each mode appeared to expand and/or populations of cells containing smaller alleles were lost, such that by the latest passages (16–20), the major repeat length in all of the lines was >2000 repeats.

Reduced survival in DM cell lines

The DM cells rarely reached a confluent state (Fig. 1B) and were more mobile (time-lapse analysis) than the controls (data not shown). All the DM cell lines showed a reduced proliferative capacity and a longer doubling time when compared with the controls (Fig. 1C). The rate of growth of the DM cell lines declined with increasing time in culture until a point was reached when there were too few cells remaining to start the next passage. To determine whether reduced growth or increased death was responsible for the differences in doubling time, we carried out additional growth experiments. Surprisingly, over a short time period, the fold stimulation of growth by 10% FCS was similar across all the cell lines and, if anything, was higher in the DM cells (Fig. 1D). The data indicate that it was not the capacity to proliferate that was responsible for the differences in doubling time between the controls and DM cells but rather a difference in cell survival. This was confirmed by the observation that lactate dehydrogenase (LDH) released into the medium was greater in the DM cell lines than in the controls (Fig. 1E).

DM cells express increased message for SK and reduced levels of *SIX5*

DMPK was found to be expressed in all lens cell lines, and recently we have also found message for *DMPK* in normal native human lens tissue (data not shown). The levels of message for *DMPK* and also *DMWD* (*dystrophia myotonica-containing WD repeat motif*) were not significantly affected by DM, although the mean values for the former in three of the four DM cell lines were greater than those of the control cells (Fig. 2A and B). Previous work has suggested that DM cataract is associated with a haplo-insufficiency of *SIX5* expression (6,7), and we found reduced levels of *SIX5*, both at the mRNA and protein level (Fig. 2C and G), in the DM cell lines consistent with this hypothesis.

The potassium channel *SK3* has previously been reported to be over-expressed in the muscle of DM1 patients (13,14) and we report for the first time in non-muscle tissue derived from DM1 patients, increased expression of this gene and also of *SK2* (Fig. 2E–F). There was, however, a large variation in the level of message across all the cell lines for the remaining member of the SK family, *SK1* (Fig. 2D), which was not consistent with DM.

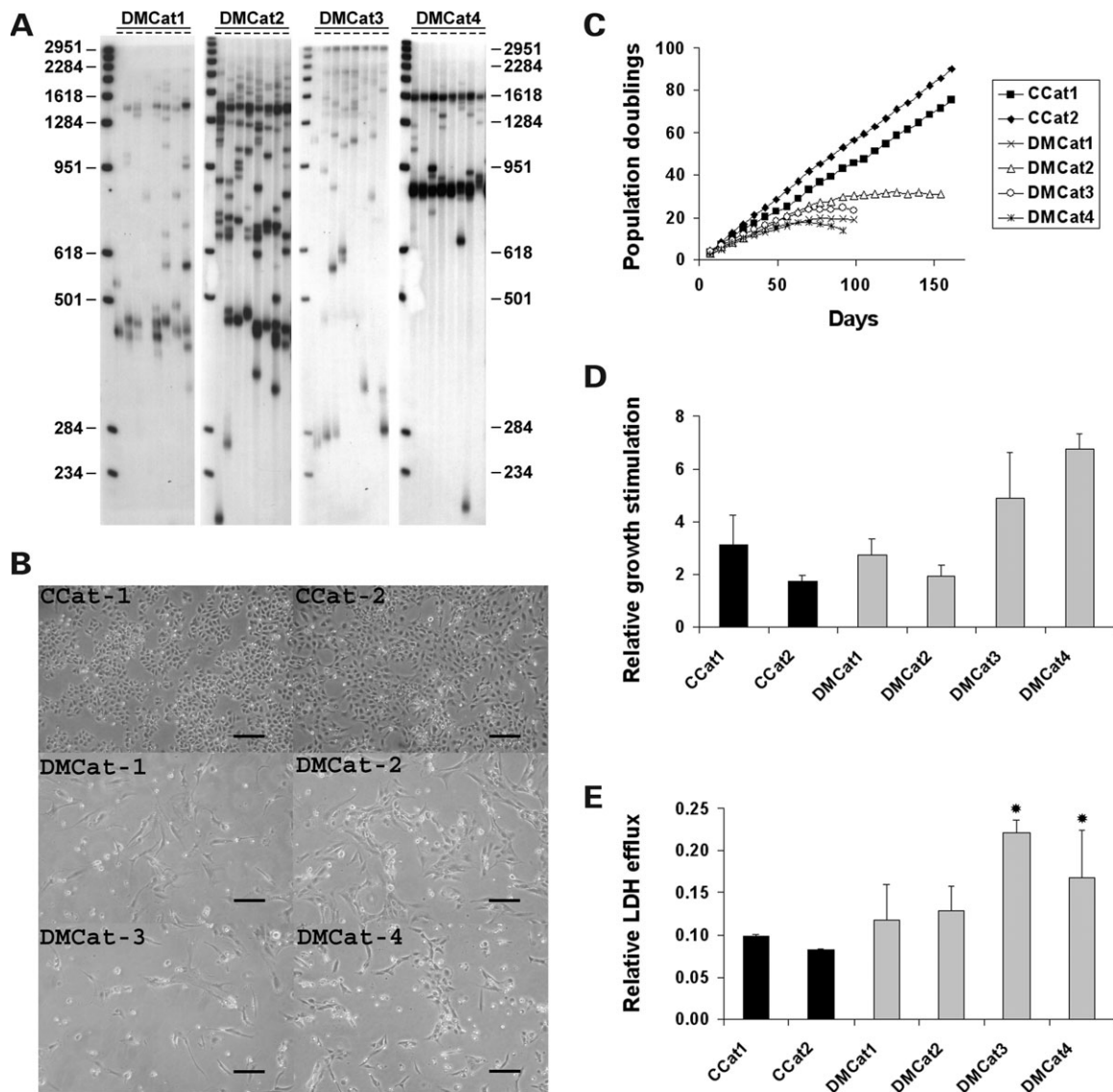


Figure 1. DMCat cell lines retain fundamental characteristics of DM. (A) CTG repeat lengths in the DM lens cell lines measured by SP-PCR. Shown are representative SP-PCR autoradiographs at passage 10. For each sample, eight reactions containing approximately 40 genome equivalents of DNA are shown. Large CTG expansions in the 3'-UTR of the *DMPK* gene were present in all the DM1 cell lines. The scale shows molecular weight markers converted into CTG repeat numbers. (B) Light microscope phase images showing cell density and morphology in control and DM cells. Cells were grown under the same conditions in 10% FCS for 3 days. The scale bar represents 100 μ m in each case. The images were obtained using a Nikon Eclipse microscope with $\times 10$ phase objective and captured with a Nikon Coolpix 995 digital camera. (C) Cumulative cell population doublings in control and DM cells. Cells were grown in 75 cm² flasks in EMEM supplemented with 10% FCS. The initial seeding density was 10^6 cells/flask and the cells were counted weekly using a haemocytometer. Note that the DM cells show a decline at later passages because fewer cells were harvested than seeded. (D) Stimulation of growth by the addition of 10% FCS. Cells were grown in 35 mm diameter culture dishes (25×10^3 cells/dish) in EMEM with or without the addition of 10% FCS for 48 h. The cells were lysed and cell growth was measured using the BCA method to analyse total protein in the lysate. The results are expressed as the growth in 10% FCS relative to the growth in serum-free medium. $n = 4$. Error bars represent the SEM with $P > 0.05$ in each case. (E) LDH released into the medium as a measure of cell death. Cells were grown in 35 mm diameter culture dishes (25×10^3 cells/dish) in EMEM for 48 h. The LDH released into the medium was measured using a colorimetric assay (Roche Diagnostics GmbH) and the values obtained were normalized to the total LDH content of the cells released at the end of the experiment. $n = 4$. Error bars represent the SEM; * $P < 0.05$.

Potassium influx and efflux kinetics

One possible explanation for the increased death rate could be a failure in the maintenance of intracellular ionic homeostasis especially under stress. We, however, found no consistent difference in the resting influx of potassium, which is partly an active process, between the DM and control cells (Fig. 3A). The resting

influx kinetics of both cell types measured over a 2 h period could be fitted by a single exponential, with rate constant (k) equal to $8 \times 10^{-3} \text{ min}^{-1}$. The influx data for the six cell lines investigated are given in Figure 3B and show a remarkable constancy of influx. Furthermore, the proportion of the influx that was inhibited by the specific Na^+, K^+ ATPase inhibitor ouabain (1 mM) was $\sim 50\%$ in all cell lines (total data not shown; but see

Table 1. Major repeat length modes in DM1 lens cell lines

Passage	DMCat1 (67 years)		DMCat2 (36 years)		DMCat3 (31 years)		DMCat4 (69 years)		
	7	10	5	10	5	10	5	10	18
Repeat length modes (CTG repeats)	~1700 ~1400, 430	>3000 ~1500, 450	~1400 420	~1400 450	>2000	>2000 ~3000 ~2100	>2000 ~1600 ~1000	>3000 ~1600 ~880	>2000 ~1700 ~920

Fig. 3A for an example). Apamin, a specific inhibitor of SK channels, had no effect on the resting potassium influx kinetics (data not shown).

K⁺ efflux is a passive process and the rate constant in unstimulated cells was ~8 × 10⁻³ min⁻¹ (Fig. 3C–E) in both control and DM cells. As this value is the same as the influx rate constant (Fig. 3A), it proves that the cells were in steady state with respect to K⁺ transport during the experimental time period. These data show that there is a remarkable agreement between the potassium transport characteristics of our cells and previously published data from cultured lens cells (17). The control and DM cells responded to the addition of a range of G-protein-coupled receptor agonists (ATP, acetylcholine, histamine and thrombin) that all release calcium in lens cells (18,19) with an increase in K⁺ efflux (Fig. 3C). There was, however, no obvious influence of DM on the magnitude of the responses, and apamin had no effect on either the resting or the stimulated efflux rate constant (Fig. 3C). Increasing intracellular Ca²⁺ with ionomycin produced a more than 10-fold increase in the rubidium efflux in all cell lines (Fig. 3D and E). In ionomycin experiments, the K⁺ efflux characteristics of the DM cells differed from the control cells in two important respects. First, the time taken to reach the peak level of efflux was less (5 min) than that for the control cells (10–15 min). Secondly, apamin inhibited the initial phase of the Ca²⁺-stimulated K⁺ efflux to a greater extent in the DM cells than in the controls (Fig. 3D–F).

Effect of high Ca²⁺ on cell survival

Ionomycin is an irreversible calcium ionophore which will result in prolonged high levels of intracellular calcium, and a consequence of this is the activation of calcium-activated proteases, such as calpain, and consequent loss of protein from the lens (20). The kinetics of ionomycin-induced LDH release are remarkably similar to those of K⁺ efflux in that a more rapid release occurs in DM cells, and apamin only reduces the loss in DM cells (Fig. 4A and B). Furthermore, apamin helps preserve the total protein content of DM cells, following ionomycin-induced stress with little effect on the protein content of CCat cells (Fig. 4C).

DISCUSSION

Why the development of cataracts is such a characteristic feature of DM is unclear. Most puzzling is their presence in the most mildly affected late onset patients, who inherit relatively small expansions (<100 CTG·CAG repeats) and who typically do not develop significant skeletal muscle symptoms. This might be explained by a particular sensitivity of lens cells to even small CTG repeat expansions. Interestingly, in contrast to skeletal muscle symptoms, and despite inheriting relatively large expansions, congenitally affected children do not present with cataracts at birth and do not usually develop them until after 10 years of age (1). These data therefore suggest that CTG·CAG expansions do not affect the normal development of the lens, but rather the ability of the lens to regulate post-natal stresses.

To our knowledge, no data have previously been presented on the repeat length present in the lens cells of DM patients.

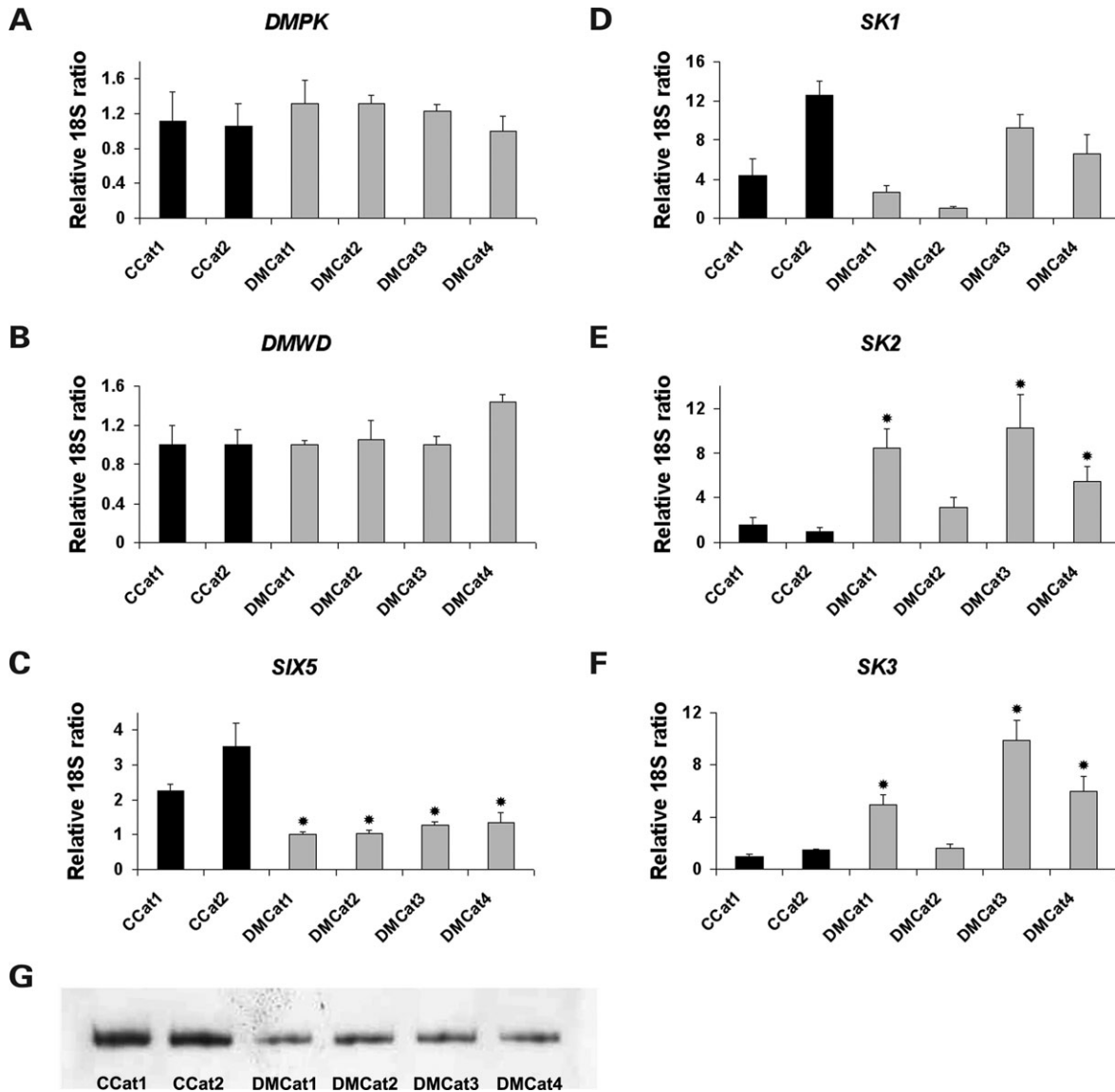


Figure 2. DM cells express increased message for SK channels but reduced levels of *SIX5*. (A–F) QRT-PCR analysis of mRNA expression from (A) *DMPK*, (B) *DMWD*, (C) *SIX5*, (D) *SK1*, (E) *SK2* and (F) *SK3*. Cells were grown for 4 days in 10% FCS and total RNA extracted. Details of the PCR primers used are given in the text. In all cases, expression is given as a ratio of 18S expression and values are normalized to the sample with the lowest expression within each gene. $n = 4$. Error bars represent the SEM; * $P < 0.05$. (G) Immuno blot analysis of *SIX5* protein. Twenty micrograms of total soluble protein was loaded onto the gel and the proteins separated by electrophoresis. A protein band corresponding to *SIX5* (100 kDa) was detected using the monoclonal antibody (MANSIX5 A3).

However, a limited analysis of the CTG-CAG repeat length present in the RT-PCR products of the *DMPK* gene from cataract material from DM1 patients has been presented (4). Although these data are potentially susceptible to RNA polymerase errors/reverse transcriptase artefacts, they data appear to indicate that lens epithelial cells carry larger expansions than present in peripheral blood leukocytes. Likewise, the data we have presented here (Table 1) defining the length of the expanded alleles in the earliest passages of cultured lens cell lines derived from DM patients strongly suggest that the lens epithelial cells from which these lines were derived contained very large expanded CTG-CAG alleles *in vivo*. The presence of very large expansions in these lens cell lines from patients with relatively small expansions in their blood DNA strongly

suggests that the DM1 repeat is indeed highly unstable in lens cells, consistent with the theory that tissue-specific somatic mosaicism may be a major contributing factor in the development of cataracts in otherwise mildly affected DM patients.

It has previously been suggested, from data obtained in the muscle system, that the increased CTG expansion modulates expression of the three genes *DMPK*, *DMWD* and *SIX5*. Although, *DMPK* expression has been found in human and animal lenses (4,7,8), there remains some controversy as to the true extent of its expression in the human lens (9). Here we show expression of *DMPK* in lens cell lines, using the highly sensitive quantitative RT-PCR (QRT-PCR) technique. Although not statistically significant, the level of expression was higher in the DM cells than in the controls (Fig. 2A). Interestingly, transient

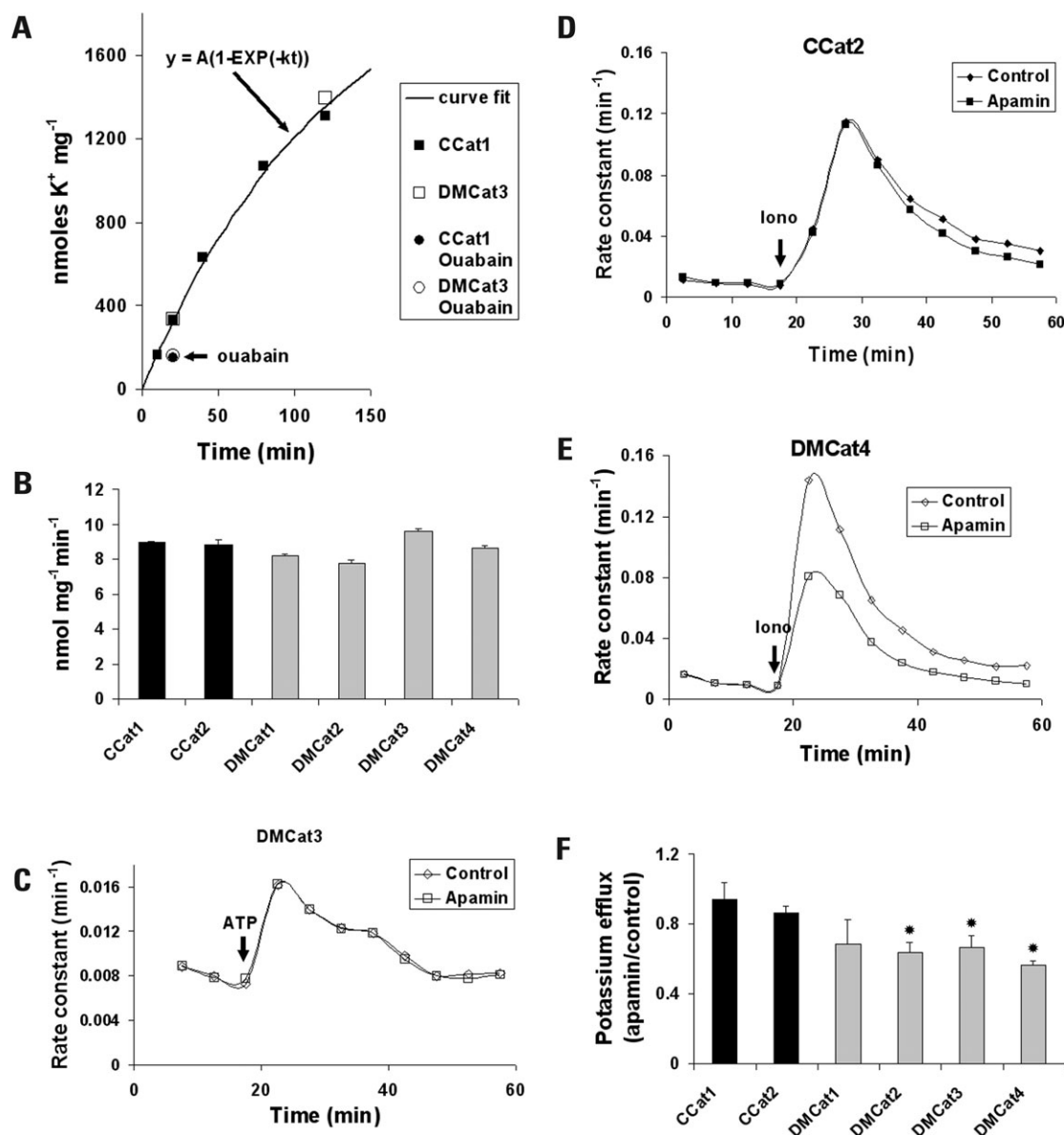


Figure 3. DM and CCat cells have similar resting K^+ flux kinetics but ionomycin reveals importance of increased SK expression. **(A)** The time course of K^+ influx. DM and CCat cells were incubated in medium labelled with ^{86}Rb and uptake was measured. Each point represents the average of two dishes of cells. A curve was fitted using the equation displayed, where $A = 2200 \text{ nmol } K^+ \text{ mg}^{-1}$ (the maximum steady-state K^+ content) and $k = 8 \times 10^{-3} \text{ min}^{-1}$ (the influx rate constant). Representative influxes measured in the presence of ouabain (1 mM for 20 min) are indicated. **(B)** Potassium (^{86}Rb) influx rates measured in a separate series of experiments over a 2 h period. $n = 4$. Error bars represent the SEM. Note that as in (A), there was no significant difference in the behaviour of control and DM cell lines. **(C)** ATP (10 μM) induced stimulation of potassium (^{86}Rb) efflux measured in DMCat-3 in the presence and absence of apamin (1 μM). Rate constant calculation is described in Materials and methods. Addition of ATP is indicated by an arrow. ATP induced a similar increase in K^+ efflux in control cells, and again apamin had no effect (data not shown). Note that for (C), (D) and (E), each point was generated from the average of two dishes. **(D and E)** Potassium efflux measurements showing the effect of adding the Ca^{2+} ionophore, ionomycin (10 μM), indicated by an arrow, in the presence and absence of apamin (1 μM). Note that in this case, the behaviour of the CCat cells and DM cells are quite different. **(F)** Effect of apamin (1 μM) on the initial K^+ efflux rate constant (k) measured 5 min after the application of ionomycin. * $P < 0.05$; $n = 4$. Error bars represent the SEM. The data were generated from control and apamin experiments illustrated in (D) and (E). Note that apamin had little effect on the CCat cells but significantly reduced the efflux rate constant in three of the DMCat cells.

transfection of lens cells with DMPK induces apoptotic-like blebbing of the plasma membrane (21), and overexpression of DMPK in transgenic mice leads to DM-like symptoms accompanied by reduced tolerance to stress, Ca^{2+} overload and muscle fibre degeneration (22). Previous work has suggested that cataract formation is associated with a haplo-insufficiency of SIX5

expression in the lens (6,7,9) and we found reduced levels of SIX5, both at the mRNA and protein level, in the DM cell lines consistent with this hypothesis. The conclusion we draw from the expression data is that although a reduced level of SIX5 could contribute to the DM phenotype observed in the cell lines, so could the expression of mutant RNA from the DMPK

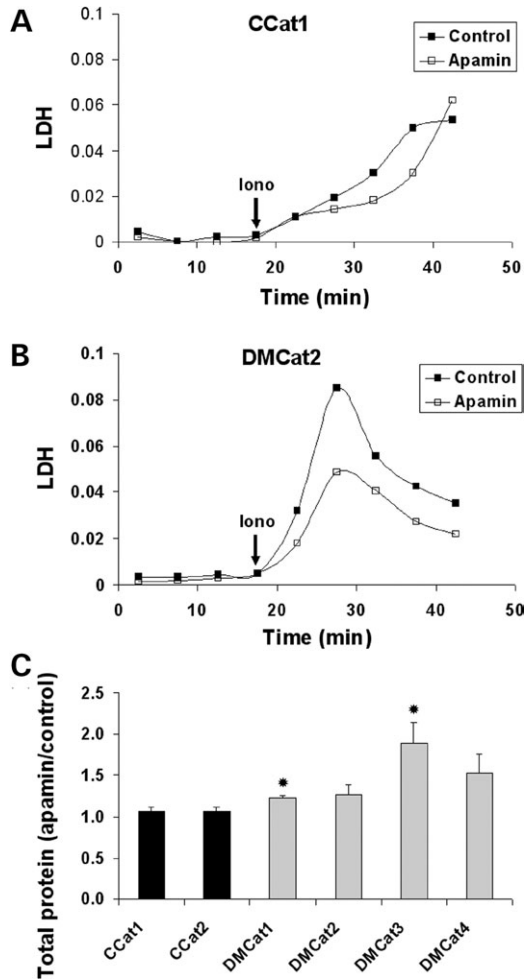


Figure 4. Apamin inhibits ionomycin-induced cell death in DM cells. (A) LDH released into the medium was measured at 5 min intervals by replacing with fresh medium. Ionomycin (arrowed) induced a gradual stimulation of LDH release, which was relatively unaffected by apamin. Each data point was derived by taking the mean of two separate experiments. (B) Ionomycin-stimulated LDH release measured in DM cells under the same conditions as in (A). In this case, ionomycin induced a more rapid increase in LDH loss, which was inhibited by apamin. (C) Total protein (BCA assay) measured at the end of experiments carried out under the same conditions as (A) and (B). Apamin had a protective effect on the final protein content of DMCat cells but not the CCat cells. Note that in each case, the mean values for the DMCat cells are above those of the CCat cells but only DMCat-1 and 3 attained statistical significance. * $P < 0.05$; $n = 3$.

locus, and this cannot, therefore, be discounted as a factor leading to cataract in the native lens.

Over long-term culture, the control cell lines (CCat1-2) showed markedly different population-doubling characteristics from the DM cells and in fact the DM cells showed a decline at later passages because fewer cells were harvested than seeded (Fig. 1C). It was therefore surprising to find that all the cell lines were stimulated to grow, over the short term, to a similar extent by serum (Fig. 1D). However, the DM cells released more LDH in culture than the controls (Fig. 1E), indicating that it is not a reduction in proliferative capacity but rather a higher death rate that explains the lengthening doubling times in the DM cell lines. A similar observation

concerning robust proliferation but increased cell death has also been suggested as a mechanism to explain the behaviour of cultured lymphoblastoids from DM patients (23).

One possible explanation for the increased death rate could be a failure in the maintenance of intracellular ionic homeostasis, especially under stress. Na^+/K^+ ATPase plays a critical role, and the $\alpha 1$ subunit has been suggested as a likely target for regulation by SIX5 (24). A reduced Na^+/K^+ ATPase activity has been found in muscle cells and erythrocytes (25,26) from DM patients. We, however, found no consistent difference in the resting influx of potassium between the DM and control cells. Furthermore, the proportion of the influx that was inhibited by the specific Na^+/K^+ ATPase inhibitor ouabain (1 mM) was approximately the same in all cases, indicating that there was little difference in the resting activity of the Na^+/K^+ ATPase between the control and DM cell lines. The present data support Klesert *et al.* (6), who found that expression of the Na^+/K^+ ATPase $\alpha 1$ subunit was unchanged in a heterozygous SIX5 knockout mouse model.

Here we report for the first time an increased expression of SK channels in human lens cells derived from DM1 patients (Fig. 2D–F), and significantly, we also report alterations in the functional activity of SK channels in these cells. It is unlikely that the increased expression arises from a negative regulation by SIX5, as there was no correlation in the individual cell lines between SK and SIX5 expressions Fig. 2. Furthermore, Sun *et al.* (27) have made a thorough investigation of the potential transcription factor-binding sites in the *SK3* gene, and no site for SIX5 was found. We show that increased expression of SK channels does not alter either influx or efflux of potassium in unstimulated cells or the efflux rate after physiological stimuli (e.g. ATP and thrombin) (Fig. 3A–E). Further we show that the highly specific inhibitor of SK channels, apamin (11), does not alter the resting rate constants for potassium flux or the G-protein-stimulated effluxes (Fig. 3C–E). A supra-stimulation by ionomycin did differentiate between control and DM cells, and under these conditions, apamin had a greater inhibitory effect on the latter (Fig. 3D–F). It should be pointed out that apamin does not reduce the ionomycin-stimulated efflux to the control rate, so other potassium-transporting mechanisms play a part in the process. These could include other classes of Ca^{2+} -activated potassium channels as well as other transporters (28). However, both the more rapid efflux of K^+ and the greater degree of inhibition by apamin in the DM cells, after intracellular calcium was increased by ionomycin (Fig. 3D–F), can be correlated with the level of SK expression. In both lens and muscle, the relationship between SK expression, Ca^{2+} and tissue pathology shows great similarities and intriguing differences. In muscle, unlike the lens, SK is normally not expressed in the adult but is expressed at high levels in DM-affected muscle cells (13,14). Furthermore, injection of apamin into affected muscle reduces the electrical activity associated with myotonia in DM patients and it is this aspect that has received most attention (12). We now suggest that the muscle-wasting aspect of the disease is also associated with increased SK channel activity. As far as the lens is concerned, this cell fragility in the presence of increased expression of SK channels could explain the reduced cell density of anterior epithelial cells in DM1 cataracts (4).

Cortical cataract is associated with a depolarization of membrane potential (29), and activation of SK channels would tend to counteract such a change and stabilize the voltage. As cortical cataract occurs earlier in life in DM, it suggests that lenses of DM patients are more prone to stress, and perhaps an increased expression of SK channels is an attempt by the lens to regulate additional depolarizing stresses. Previous studies with normal native tissue have shown that increased expression of SK channels correlates with quiescent anterior cells rather than dynamic, dividing equatorial cells (15). Interestingly, it is the anterior cell population that is more directly subject to depolarizing stresses such as exposure to UV light and temperature fluctuation. Cells with a superabundance of SK channels would respond to a rapid increase in internal Ca^{2+} , with a much larger change in potassium conductance leading to a greater loss in internal K^+ . If this loss was not compensated for by an increase in pump activity, then such a loss could lead to osmotic fragility or, indeed, ultimate cellular disintegration through potassium deficiency-induced apoptosis. Remillard and Yuan (30) point out that enhanced potassium efflux is an essential mediator of not only early apoptotic cell shrinkage, but also of caspase activation and DNA fragmentation. Interestingly, ionomycin induces a faster, larger increase in K^+ efflux from DM cells with a concomitantly greater loss of protein. This loss in DM cells is inhibited by apamin. More physiologically appropriate stimuli of cytoplasmic Ca^{2+} via G-protein-coupled receptor activation are accompanied by increases in K^+ efflux that are very similar in control and DM cells, and in neither case is the efflux inhibited by apamin. Again this suggests that DM cells behave normally to physiological stimuli, but are unable to deal with unscheduled stress. There is also evidence that DM eyes may be subject to additional stresses. For example, the lower intraocular pressure is accompanied by a higher rate of inward leakage of protein material. As this appears to originate from the uvea (31), it is likely to contain growth factors, cytokines and other Ca^{2+} -mobilizing agents. The effects of such stresses accumulate with age and so could explain the relatively late onset of DM cataracts compared with certain catastrophic, congenital cataracts that are observed at birth (32).

MATERIALS AND METHODS

Cell lines

A total of six cell lines were derived by SV40 transformation of lens capsulorhexis specimens obtained immediately following cataract surgery. The two control cell lines were obtained from patients aged 28 and 43 years, whereas four DM cell lines were generated from patients aged from 39 to 69 years. Cells on capsulorhexis samples were immortalized using adenovirus, 12 simian viruses and 40 hybrid viruses. Virus was produced in African green monkey CV1 cells and stored at -80°C as culture supernatant. Virus was added directly to the capsule fragment upon arrival in the laboratory, and the cells were monitored for growth over a 2–3 week period. Once the cells had migrated and covered by $>50\%$ of the area of a 24 well culture plate, they were trypsinized and transferred into a single well of a six well plate. The cells were

allowed to grow to confluence and then passaged into a 25 cm^2 flask. Cells were increased in number by passaging into larger flasks until sufficient cells could be frozen (typically passage 5). Fleming *et al.* (33) have previously employed a similar technique to generate the HLEB3 cell line from primary cultures of human lens cells. This much-used cell line expresses crystallin proteins and we have also confirmed their expression in all our cell lines (data not shown).

Cell growth analysis

Cells were brought up from frozen and cultured in 75 cm^2 flasks and EMEM-supplemented with 10% FCS at 35°C and 5% CO_2 . Cells were passaged weekly at a seeding density of 10^6 cells per flask and counted using a haemocytometer. Stimulation of growth by 10% FCS was assessed in cells grown in 35 mm diameter culture dishes (25×10^3 cells/dish) in EMEM. After 48 h, the cells were lysed and the protein content of the lysate was determined using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) according to the manufacturer's instructions. Cell death was quantified by measuring LDH release into the medium, using a colorimetric method (Roche Diagnostics GmbH, Germany). Cells were grown in 35 mm diameter culture dishes (25×10^3 cells/dish) in EMEM for 48 h. The medium was changed daily and the LDH released was quantified and normalized to the total LDH content of the cells released at the end of the experiment by the application of 1% Triton.

DNA analysis

Genomic DNA was extracted using a DNeasy Tissue Kit (Qiagen, UK) by following the manufacturer's instructions. Small Pool PCR analysis of repeat length variation at the DM1 locus was performed as described previously, using primers DM-A and DM-BR (2,34).

Quantitative real-time PCR

Gene expression levels were measured by QRT-PCR (Taqman®) using gene-specific primers and probes. For *DMPK* and *DMWD*, we used Assays-on-Demand (Applied Biosystems). The assay identifiers are Hs00189385_m1 (*DMPK*) and Hs00412888_m1 (*DMWD*). For *SIX5*, we used primers which amplified a product that included the boundary between exons 2 and 3 (Assays-by-Design, Applied Biosystems). The primer sequences are as follows: forward—GCTGCCTTCGGCCACT; reverse—GCCACACCCGTCAC GAT. The primer sequences for *SK3* have already been published (15). Cells were cultured in 60 mm diameter plastic culture dishes in EMEM supplemented with 10% FCS for 4 days. Total RNA was extracted using a Qiagen RNeasy Mini kit (Qiagen) by following the manufacturer's instructions. Reverse transcription was performed with $1\text{ }\mu\text{g}$ of RNA, using SUPERScript II and Random Primers (Invitrogen), according to the manufacturer's protocols. Amplification was carried out using the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems) according to the manufacturer's instructions. In a total reaction volume of $25\text{ }\mu\text{l}$, 5 ng of cDNA was used for the genes of

interest. For *I8S* expression, 1 ng of cDNA was used. Forward and reverse primers were added at a final concentration of 200 nM. A Mastermix (Applied Biosystems) containing all the components necessary for the PCR reaction was used. All QRT-PCR expression levels are presented relative to the *I8S* value.

Western blotting

Cells were grown in 75 cm² flasks for 5 days. The medium was removed, the cells were washed with PBS and lysed in 1 ml of M-PER lysis buffer (Pierce) plus 10 µl/ml of proteinase inhibitor cocktail (Halt, Pierce) and 10 µl/ml EDTA (5 mM) according to the manufacturer's instructions. The lysates were spun at 16×10^3g for 10 min at 4°C and the supernatants frozen at -80°C until required. The protein content of the soluble fraction was determined using a BCA (Pierce) according to the manufacturer's instructions. Equal amounts of protein (19 µg) were loaded into each well of a 7% SDS-PAGE gel for electrophoresis and transferred onto polyvinylidene difluoride membrane, using a semidry transfer cell. Proteins were detected using a chemiluminescent blot analysis system (ECL+; Amersham Biosciences, Amersham, UK). Monoclonal antibodies for SIX5 (MANSIX5-A3) were obtained from Glen Morris (Centre for Inherited Neuromuscular Disease, Oswestry, UK) and were used at a dilution of 1:100 (35).

Potassium influx/efflux measurements

⁸⁶Rubidium (⁸⁶Rb) was used as a tracer ion to measure potassium fluxes. Cells were grown in 35 mm culture dishes for 72 h in EMEM supplemented with 10% FCS at 35°C and 5% CO₂. At the end of this period, the cells had not reached a confluent state (Fig. 3). This is important because after a longer period, the faster growing controls reached confluency before the DM cells. For influx measurements, cells were incubated in medium labelled with ⁸⁶Rb (c55 kBq/ml) for up to 2 h at 35°C (5% CO₂). Cells were washed three times with ice-cold medium, and ice-cold trichloroacetic acid (5%) was added. An aliquot of the TCA was sampled after 30 min incubation and the radioactivity measured by scintillation counter.

For the efflux investigations, the cells were loaded overnight in medium labelled with ⁸⁶Rb (c55 kBq/ml) and washed three times with warmed EMEM (35°C) before the start of the experiments to remove excess ⁸⁶Rb. To record the efflux, the medium was removed, retained for later analysis and replaced with fresh pre-warmed medium at 5 min intervals for a total time of 1 h. At the end of the experiment, 1 ml of ice-cold TCA (5%) was added and the cells were incubated for 1 h at RT. After the TCA was removed, 0.5 ml of 0.1 M NaOH was added and incubated overnight at 4°C. A sample of the lysate was collected for protein analysis (see western blotting section).

Efflux rate constants were calculated as described by Delamere and Duncan (36):

$$k = \frac{\ln(A_{t1}) - \ln(A_{t2})}{t_2 - t_1}$$

where k is the efflux rate constant and A_{t1} and A_{t2} are the activities in the lens (counts/min) at times t_1 and t_2 , respectively.

Statistical analysis

Statistical analysis was performed by two-tailed *t*-test. Significance was considered at $P \leq 0.05$.

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